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ULTRASTRUCTURAL EVALUATION OF THE RETINA IN RETINOPATHY OF PREMATURITY AND CORRELATIONS WITH VITAMIN E THERAPY

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NAVAL AEROSPACE MEDICAL RESEARCH LABORATORY
PENSACOLA, FLORIDA

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SUMMARY PAGE

THE PROBLEM

Retrolental fibroplasia, or retinopathy of prematurity, is the cause of moderate vision loss in more than 1300 infants born prematurely each year. Approximately 250 premature infants suffer some form of permanent blindness from this disease.

FINDINGS

A clinical study to evaluate the efficacy of vitamin E in reducing the incidence or severity of retinopathy of prematurity (ROP) was performed at Texas Children's Hospital in Houston, Texas. The study concluded that the vitamin therapy may have been effective in reducing the most serious grades of ROP, but did not reduce the incidence of the non-cicatricial grades of the diseases.

This paper summarizes the results of a histological study performed on ocular tissue obtained from six premature infants who died during the course of the clinical study. The histology collaborated existing theories of angiogenesis (vascular development) as well as shunt formation in the latter grades of ROP. The evaluation revealed a developmental dichotomy in the spindle cell morphology of the nerve fiber layer between control and experimental infants. The results of the histological evaluation are by no means conclusive, however they do provide new information about the development of the disease at the ultrastructural level.

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Ultrastructural evaluation of the retina in retinopathy of prematurity and correlations with vitamin E therapy

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ABSTRACT

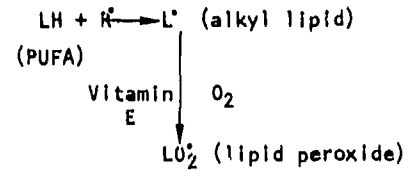
Histological evidence of retinal damage associated with the clinical observation of Retinopathy of Prematurity (ROP) grade III was documented in preterm infants receiving the minimum dosage of vitamin E recommended by the American Academy of Pediatrics (5 mg/kg/day), and exposed to high concentration/duration of oxygen at birth. Matched infants that were provided a higher oral dosage of vitamin E (100 mg/kg/day) did not develop the serious grade of retinopathy (grade III) (1,2). In this paper cytological correlates are described which substantiate pre-existing theories concerning the pathological changes associated with the development of the disease at a light microscopic level. Moreover, observations made at the electronmicroscopic level permit distinctions to be made concerning the newly formed retinal vessels, in treated versus non-treated infants, that have not been noted in the history of this disease. These retinal distinctions suggest that vitamin E may be efficacious in reducing the severity of ROP. Lastly, a mechanism is suggested for the action of vitamin E in reducing the severity of ROP.

INTRODUCTION

Retinopathy of Prematurity (ROP) is a blinding eye disease of premature infants who are administered high levels of therapeutic oxygen. Ashton (3) has described the progression of ROP as the result of aberrant acceleration of the normal process of "retraction" which involves withdrawal and absorption of primitive endothelial cells during retinal vascular development. Through the process of retraction the blood is shunted and at this time there is vascular differentiation into artery, vein, and capillary.

It has been hypothesized (4) that in the hyperoxic condition the existing capillaries are partially (or totally) closed and circulation is maintained by the development of an arteriovenous "shunt." The newly developed vessels apparently do not have the structural integrity of the normal retinal vasculature since hemorrhage and ultimately retinal detachment may ensue. The obliteration of the capillary bed precedes the formation of the shunt with tortuous vessels feeding it. Ashton (4) and Foos (5) have described the region outside the shunt as a "vanguard" of invading mesenchyme and primitive endothelial cells and the region inside the shunt as a "rearguard" of normal developing retinal vasculature. Retinal vasoproliferation (aberrant overgrowth of retinal vessels) has been described by Ashton as "a non-specific response to vasoobliteration and is not itself directly due to oxygen." We have recently reported evidence that there are other contributing clinical factors such as low birthweight and gestational age, intraventricular hemorrhage, and sepsis (1,2) that predispose the retinal vasculature to damage by oxygen, due to its increased demand in these conditions.

Vitamin E has been suggested as a potential suppressant of ROP since 1949 (6). It has been proposed that vitamin E's principal biological role is as an antioxidant, acting within membrane systems, and that lipid peroxide free radicals ($LO_2^{\cdot}$) that are generated in an oxygen rich environment may be disposed of principally by interaction with vitamin E (7).



Based on some of the above, a clinical study was initiated in November of 1979 at Texas Childrens Hospital in Houston, Texas. The intent of dietary manipulation with vitamin E (a strong antioxidant) was to reduce or eliminate the most severe

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grades of this disease. This study (1,2) concluded that there was a significant difference in the development of ROP between vitamin E-treated premature infants (administered 100 mg/kg/day, orally) versus non-treated premature infants (administered 5 mg/kg/day, orally). During the course of the clinical study, 33 infants died and the guardians of 7 neonates consented to complete autopsy with immediate whole-eye donation. Six of these cases are reported here. The seventh was an infant of a diabetic mother and was not considered in this histological study. This study is the first attempt to document the retina of infants with ROP with either low or supplemented vitamin E at the ultrastructural level. Subsequent analyses based on the findings of this study are currently underway (8).

METHODS

The eyes of the deceased infants were enucleated and immediately immersed into a freshly prepared solution of 2% glutaraldehyde/2% formaldehyde in 0.135 M phosphate buffer, pH 7.4, 5°C. The eyes were retained in fixative at ambient room temperature for one hour, then cut across the ora serrata with a razor blade and separated into anterior and posterior hemispheres and fixed for an additional twelve hours in a refrigerator. At the end of this fixation period, the eyes were placed in 0.135 M phosphate buffer. Macromorphology of the posterior eyecup was documented on a Wild 8M zoom stereomicroscope. McCormick (9) has suggested a method of grading the active stages of the disease into four categories/grades. Grade I and II, by this classification spontaneously regress, whereas grades III and IV result in some form of permanent visual loss.

4mm disks, from the nasal and temporal quadrants (tangential to the ora serrata) of the posterior hemispheres were trephined then bisected into hemidisks. The anterior hemisphere was cut into four equal pieces, and the iris separated at the filtration angle. The dissected tissue was then prepared for light and electronmicroscopy.

For light microscopy, 0.5μ sections were cut on a Reichert OMU3 microtome with glass knives, mounted on glass slides, stained with 1% Toluidine blue in 0.1% sodium borate, and photographed on a Zeiss PM III microscope.

To assay histochemically for the presence of lipid, a disk of glutaraldehyde crosslinked temporal, retina was washed in distilled water and frozen at 20°C in an International Experiment Company Cryostat. Sections (6μ thick) were cut, flattened on glass slides, dried at 60°C for 5 minutes, and stained with Oil-Red-O and counter stained with hemotoxylin.

The temporal and nasal hemidisks were sequentially sectioned through their entire thickness in order to establish the distribution and nature of spindle cells within the nerve fiber layer.

Areas were then selected for ultrastructural evaluation. Silver-gold sections (60nm) were cut from the same block faces and collected on formvar coated, single-hole grids. The sections were then stained and carbon coated. The thin sections were then observed and photographed on a JEOL 100 CX electron microscope at 80kV with a 200μ condenser and 50μ objective apertures.

RESULTS

Figure 1 compares the development of ROP in control infant (#5) and experimental infant (#6). Infant #6 never progressed beyond a grade I ROP, which spontaneously regresses to normal, whereas infant #5 developed grade III ROP which results in permanent retinal scarring.

Macromorphology

In six infants that were examined histologically (see Table 1 for clinical data), the corneas were clear, there was no evidence of anterior synechia, and the lenses were free of opacification. All eyes contained a cloudy vitreous which produced the hazy appearance of the macrophotographs (Figure 2). Macrophotographs were not done on infants #2 and #3. The clinical and histological data were organized and reported on the basis of post mortem delay. The posterior poles of the right eye from infant #1 (control, 27 weeks at death, eyes obtained 1 hour post-mortem), #4 experimental, 29 weeks

at death, eyes obtained 3.5 hours post-mortem), #5 (control, 36 weeks at death, eyes obtained 4 hours post-mortem), and #6 (experimental, 46 weeks at death, eyes obtained 6 hours post-mortem) are depicted in Figures 2A through D, respectively. Infants #1, 4, and 6 (Figures 2A, B, and D) exhibited normal fundi with optic disks showing no signs of pallor or papilledema, the retinal vasculature showing no marked dilation, tortuosity, or evidence of hemorrhage and no evidence of retinal pigmentary changes or detachment. Infant #5 (Figure 2C) exhibited fundus changes commensurate with grade III ROP. There was a complete circumferential mesenchymal shunt formation demarcating vascularized from non-vascularized retina with isolated areas of pre-retinal hemorrhage. The vasculature (not visible in the macrophotograph) of infant #5 (Figure 2C) was clinically observed to be tortuous and engorged. There was vitreal condensation artifact in infants #4, 5, and 6 (Figure 2B, C, D, respectively). There was no visible evidence of deleterious effects on the tissue from post-mortem delay at this magnification.

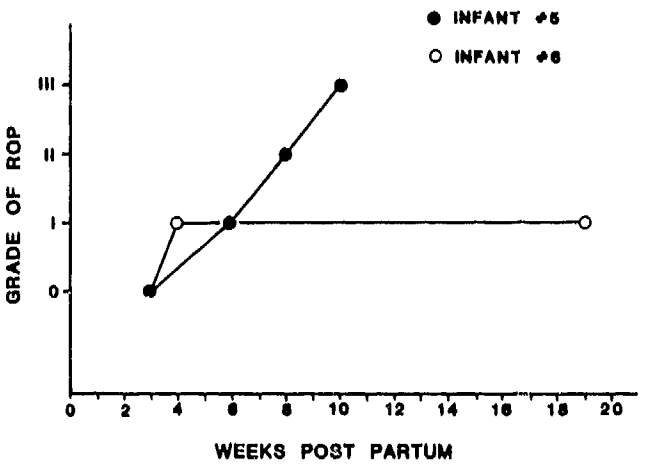

Figure 1: Progression of ROP in infant #5 (control) and infant #6 (experimental) until time of death. Ocular exams began the third week of life and were repeated weekly.

Light Microscopy of Nasal Retina

Sectors of nasal retina (Figure 3) were used as a control for the temporal retinal sections because nasal retina develops earlier embryologically than the temporal. The photomicrographs are presented in the same format and sequence throughout (control Figures 3A, 3B, and 3C for infants #1, 2, and 5;

TABLE 1: Clinical Data of Infants Whose Eyes Were in this Study.

Infant #	Birthweight (grams)	Gestational Age Average* (weeks)			O_2 Duration (FIO_2)	Time post-partem	ROP score	Hrs. Post mortem	Exp/ Cont.	Cause of Death
		GA-MD	GA-PE	GA-TV						
1	950	26	28	N/A	2 days	2 days	0	1	C	IVH***
2	1060	28	28	N/A	5 days	5 days	0	1	C	IVH
3	760	26	26	27	3.2 weeks	3.4 weeks	0	1	E	respiratory failure
4	830	26	27	N/A	2 weeks	2 weeks	0	3.5	E	"
5	625	26	N/A	N/A	7 weeks	10 weeks	3	4	C	"
6**	1030	27	28	N/A	19 weeks	19 weeks	1	6	E	"

* Gestational age was evaluated by three methods: Mother's dates (MD), Physical Exam (PE), and Appearance of tunica vasculosa lentis (TV).

** This infant developed severe Hyaline Membrane Disease by the second week of life and thereafter poor PaO_2 response to high environmental oxygen. It is known that two weeks of oxygen stress is sufficient time to induce retinal vascular damage in the premature infant. The remaining five infants had normal PaO_2 responses to environmental oxygen.

*** Intraventricular hemorrhage (IVH) is hemorrhage of the immature vasculature into the subependymal germinal matrix of the brain.

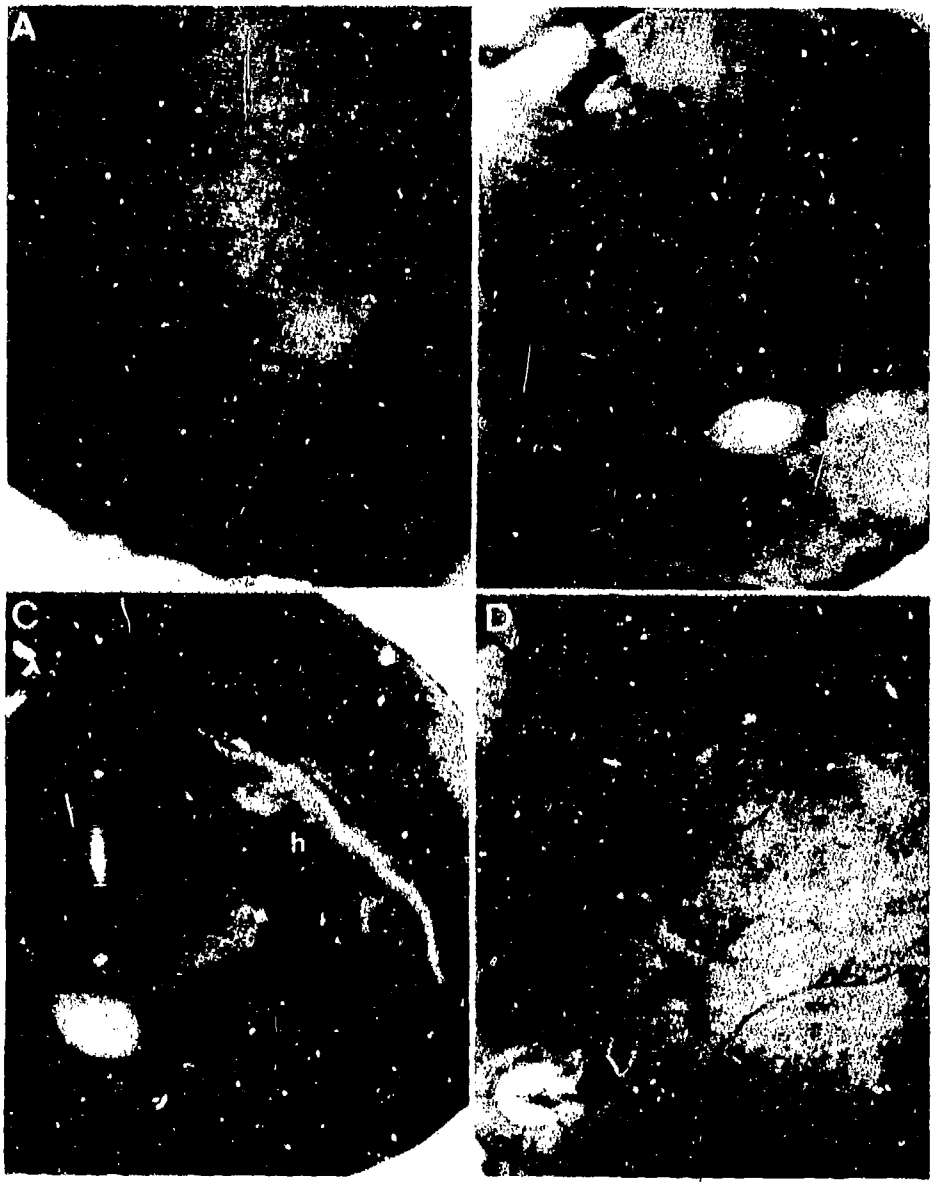

Figure 2: A light macrophotograph of the posterior pole (O.D.) from four infants in the study. Three of the infants exhibited normal fundi: Figure 2A (infant #1, control, 27 weeks at death, eyes obtained 1 hour post-mortem); 2B (infant #4, experimental, 29 weeks at death, eyes obtained 3.5 hours post-mortem); and 2D (infant #6, experimental, 46 weeks at death, eyes obtained 6 hours post-mortem). Figure 2C infant #5, control, 36 weeks at death, eyes obtained 4 hours post-mortem) depicts grade III ROP. The optic disks (◗) showed no signs of pallor or papilledema, and the retinas were intact with no evidence of pigment accumulation, hemorrhage, or detachment. There was vitreal condensation artifact (*) in Figures 2B (#4, experimental), 2C (#5, control) and 2D (#6, experimental). Infant #5 (Figure 2C, control) exhibited a complete circumferential demarcation line (arrow) and pre-retinal hemorrhage (h). (X32)

experimental-Figures 3D, 3E, and 3F for infants 3, 4, and 6).

The pigment epithelium was normal and intact in all cases. The photoreceptor outer segments were visibly intact only in infant #6 (experimental, Figure 3F). Infant #5 (Figure 3C, control) and #4

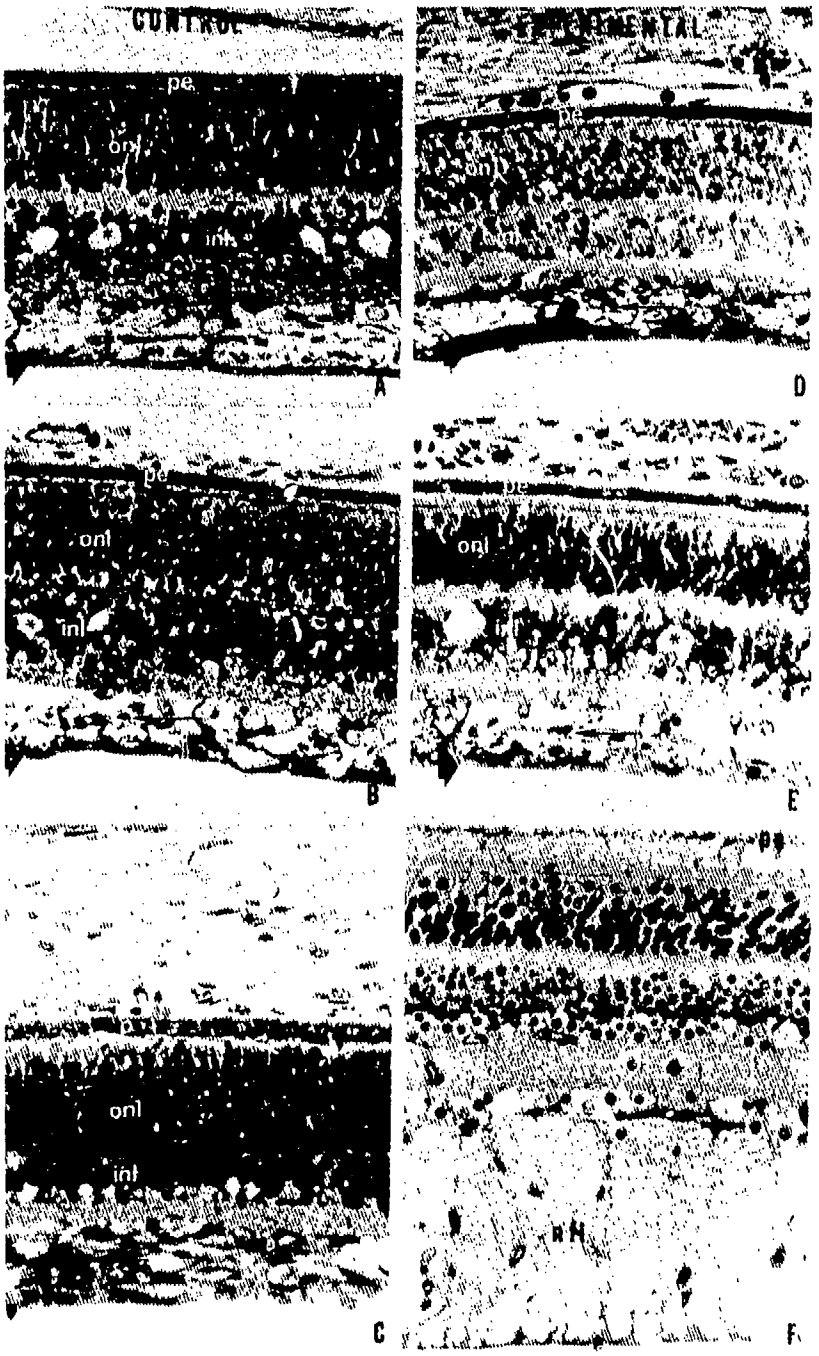

Figure 3: A light micrograph of the nasal retina from six infants. Controls are depicted in the left-hand column (infants #1, 2, and 5) and experimental in the right-hand column (infants #3, 4, and 6). The pigment epithelium (pe) is normal and intact in all cases. The photoreceptor outer segments are only present in infant #5 (control, remnant outer segment) (Figure 3C) #4 (experimental, remnant outer segment) (Figure 3E), and infant #6 (experimental, intact) (Figure 3F). The nuclear layers (onl and inl) exhibit a multi-layered appearance which is normal in thickness. The nerve fiber layer exhibits a normal population of spindle cells (⟶) in Figure 3A through E. In Figure 3F the nerve fiber layer (nfl) is much thicker and more mature (infant #6, experimental, and 46 weeks at time of death). The internal limiting membrane is normal and intact (▶). (X530)

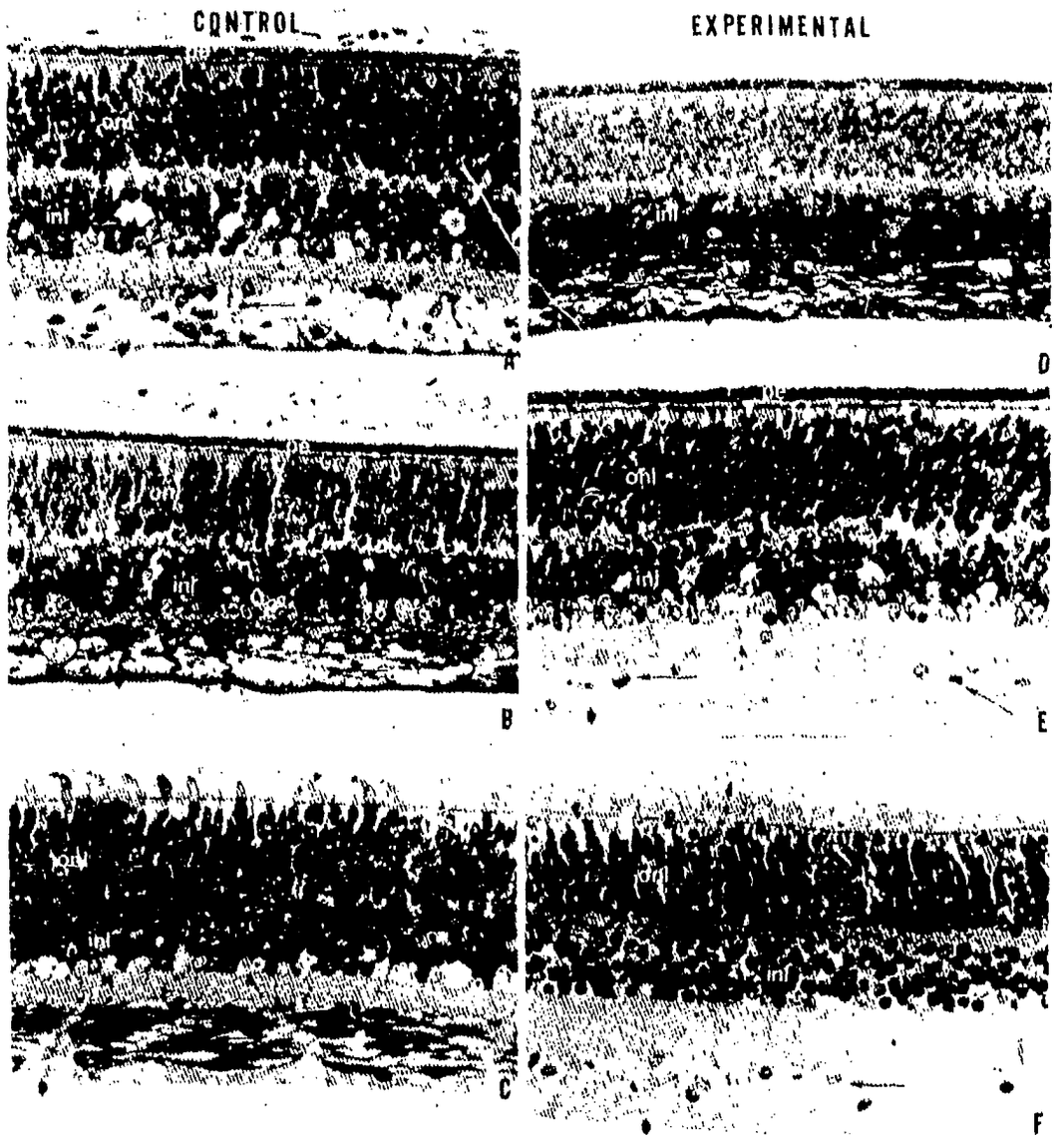

Figure 4: A light micrograph from the temporal retina of six infants. Control infants (#1, 2, and 5) are represented in Figures 4A, B, and C, experimental infants (#3, 4, and 6) represented in Figures 4D, E, and F. The pigment epithelium (pe) was artifactually separated in Figures 4C and 4F and is normal in Figures 4A, B, and E. The photoreceptor outer segments are present in Figure 4C (#5, control), and Figure 4F (#6, experimental). The external limiting membrane is intact in all cases. The nuclear layers (onl and inl) exhibit a nonpycnotic stratified appearance. Vacuoles (*) are apparent in Figures 4A (#1, 1 hr. post-mortem), 4B (#2, 1 hr. post-mortem), 4D (#3, 1 hr. post-mortem) and 4E (#4, 3.5 hrs. post-mortem). There was a noticable difference in the nerve fiber layer. The experimental infants exhibited a scanty spindle cell (⟵⟶) distribution while the most mature control infant (#5, 36 weeks at death) exhibited dense packing of spindle cells (⟵⟶) within the nerve fiber layer (see Figures 4D, E, and F for distribution of experimentals and Figure 4C for dense packing). The internal limiting membrane (◗) was intact in all cases. (X530)

Figure 5: Electronmicrographs of the temporal retina depicting the normal pigment epithelium of infant #6 (Figure 5A) (experimental, 46 weeks at death) and infant #5 (Figure 5B) (control, 36 weeks at death). The normal basal infoldings of Bruch's membrane (b) are visible as well as normal mitochondria (m) and a population of phagosomes (p). There is evidence of normal melanin accumulation (◊). Apical processes (⟵►) are seen to surround the intact rod outer segments (R) in the experimental infant (Figure 5A) whereas in the control infant (Figure 5B) the rod outer segment has been artifactually fragmented and lies at the base of the pigment epithelium amongst immature outer segments covering the apical processes. (X6000)

(Figure 3E, experimental) had remnant outer segments and the remaining three infants (#1, control, #2, control, and #3, experimental) had no visible photoreceptor outer segments remaining. The external limiting membrane was intact and the nuclear layers exhibited a stratified appearance which was normal in thickness in all cases. Infant #6 (Figure 3F; experimental) had the thickest and most mature nerve fiber layer. All other infants had less mature nerve fiber layers with spindle cells still apparent. Vacuoles were visible in the inner nuclear layer in Figure 3A - Infant #1 (1 hour post-mortem), 3B-Infant #2 (1 hour post-mortem) and 3E-Infant #4 (3.5 hours post-mortem). The

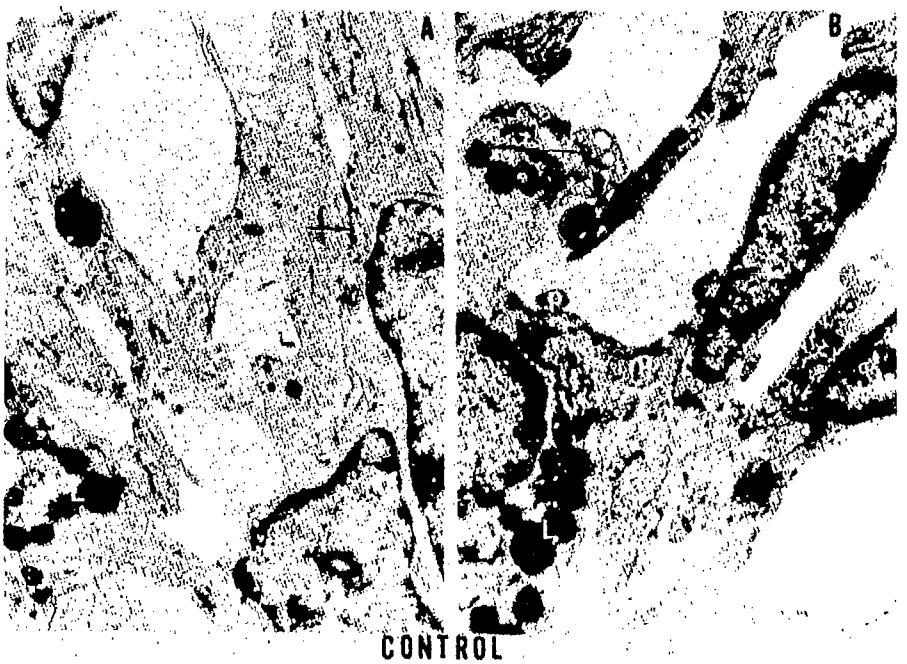

Figure 6: Electronmicrographs (Figures 6A and B) of spindle cells from the nerve fiber layer of temporal retina of infant #5 (control, 36 weeks at death, 7 weeks of oxygen administered). There is evidence of quiescent rough endoplasmic reticulum (⟶), extensive pseudopodia (p) and lipid accumulation (L), and normal appearing mitochondria (m). There is a denser population of spindle cell nuclei than seen in the experimental infants (Figures 8A and B) but junctional complexes are not visible at this magnification. (X6000)

internal limiting membrane was normal and intact in all cases.

Light Microscopy of Temporal Retina

Sections of temporal retina (Figure 4) were compared in each group (control and experimental). The pigment epithelium was normal in Figure 4A (#1, control), 4B (#2, control), 4D (#3, experimental), and 4E (#4, experimental) and artifactually separated in 4C (#5, control) and 4F (#6, experimental). Photoreceptor outer segments were visible in 4C (#5, control) and 4F (#6, experimental) and are absent in Figures 4A, 4B, 4D, and 4E (infants #1, control, #2, control, #3, experimental, #4, experimental, respectively) and the external limiting membrane was intact.

There is normal thickness of the stratified nuclear layers in all cases representing intact bipolar, amacrine, horizontal, and Muller cell populations as well as normal population of ganglion cell nuclei. In the nerve fiber layer, the spindle cells which are normally present showed no distinct spatial distributions in the control infants #1, 2, and 5 (Figures 4A, B, and C) versus the experimental infants #3, 4, and 6 (Figures 4D, E, and F). The inner limiting membrane was intact in all cases with the exception of infant #5 (Figure 4C) who showed focal breaks at the sites of pre-retinal hemorrhage (not observed in these micrographs). Vacuoles were present in the inner nuclear layer of infants #1, 2, 3, and 4 (Figures 4A, B, D, and E).

Ultrastructure of Pigment Epithelium and Photoreceptors in the Temporal Retina

Figure 5 depicts the pigment epithelium and photoreceptors of infant #6 (experimental, 46 weeks at death, eyes obtained 6 hours post-mortem) and infant #5 (control, 36 weeks at death, eyes obtained 4 hours post-mortem). The pigment epithelium was structurally sound in both cases with no dimunition of basal infoldings adjacent to

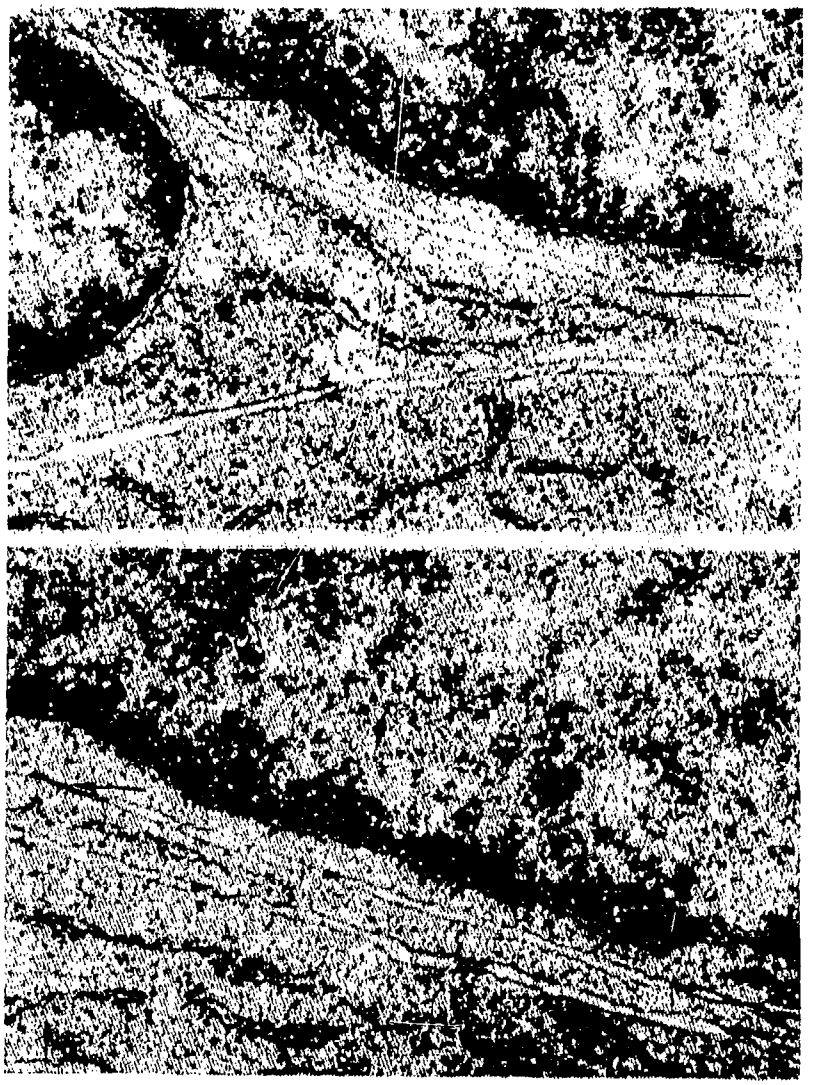

Figure 7: Electronmicrographs (Figures 7A and B) depicting gap junctions (⟶) between the adjacent spindle cells in the nerve fiber layer of the retina from control infant #5 (36 weeks at death, 7 weeks of oxygen administered). The junctional complexes may represent a means of communication between cells and may play an important role in the vascular proliferative process. (X53,000)

Bruch's membrane, normal mitochondria, no abnormal phagocytic activity evidenced by the presence of multivesicular bodies, a non-pycnotic nucleus, and a normal population of melanin-melanolysosome complexes. The apical microvillar processes are apparent in the experimental (Figure 5A) but not the control (Figure 5B) infant because of the accumulation of rod outer segment debris in the subretinal space. The photoreceptors were intact in the experimental infant (Figure 5A) whereas in the control infant the outer segments were fragmented (containing, however, intact plasma membranes) interspersed between amorphous segments at the base of the pigment epithelium (Figure 5B).

Uemura (10) and Ashton (4) have suggested that lipidic globules accumulate in the pigment epithelium, and the photoreceptor outer segments are damaged in rabbits that are exposed to high oxygen

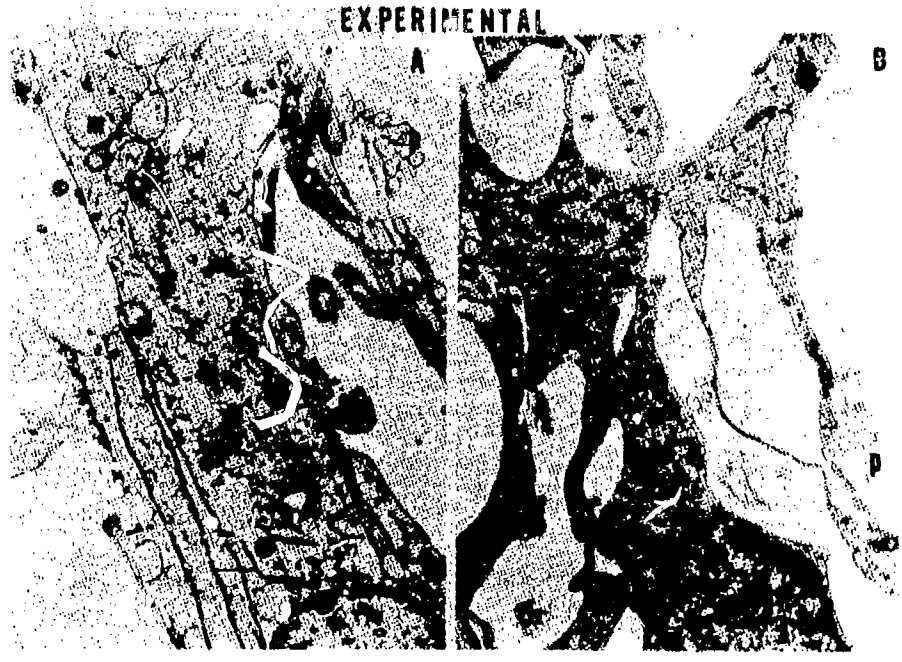

Figure 8: Electronmicrographs of spindle cells from the nerve fiber layer of the temporal retina of infants #3 (experimental, 29 weeks at death, 3 weeks oxygen administration) and #4 (experimental, 29 weeks at death, 2 weeks of oxygen administered). Infant #3 is represented in Figure 8A and infant #4 in Figure 8B. Both cases exhibit quiescent rough endoplasmic reticulum (⟶), sparse pseudopodia (p), large mitochondria (m) and rare lipid accumulation. (X6000)

(80-90%) concentrations. None of these changes was noted in the infant retinas evaluated in this study.

Structural Parameters of Spindle Cells

Isolated spindle cells from the nerve fiber layer of the temporal retina were prepared for ultrastructural evaluation. The retinas from three infants were selected (one control, and two experimental) for this evaluation. The aggregated spindle cells of the control infant (#5, 36 weeks at time of death, with 7 weeks of oxygen administered) form a stacked continuum interspersed between Muller cell foot processes. Ultrastructurally, in this control infant the aggregated spindle cells contain extensive accumulations of lipid, small mitochondria, quiescent rough endoplasmic reticulum, and extensive pseudopodia (see Figures 6A and B). Aggregated spindle cells have no basement membrane surrounding the cells and no microfilaments within the cytoplasm; the plasma membranes have a distinct separation except where gap junctions are noted (see Figure 7). The isolated spindle cells of experimental infant #3 (29 weeks at death, 3 weeks of oxygen administered) and #4 (29 weeks at death, 2 weeks of oxygen administered) are represented in Figures 8A and B, respectively. The scattered spindle cells of both of these infants contained large mitochondria, quiescent rough endoplasmic reticulum, rare intracellular lipid accumulations, and few pseudopods.

Morphological Aspects of the Shunt in Case 5 (Grade III ROP)

Figure 9 shows the dichotomy that existed in the nerve fiber layer between the vanguard region, with its stacked spindle cells, and the rearguard region, with its complete vascularization. These two regions are separated by a shunt of rolled mesenchyme entrapping erythrocytes in their extracellular space. Ultrastructurally, on the vanguard side of the shunt (Figures 10A and B), the extracellular erythrocytes are noted between spindle cells which are laden with lipid. Within the

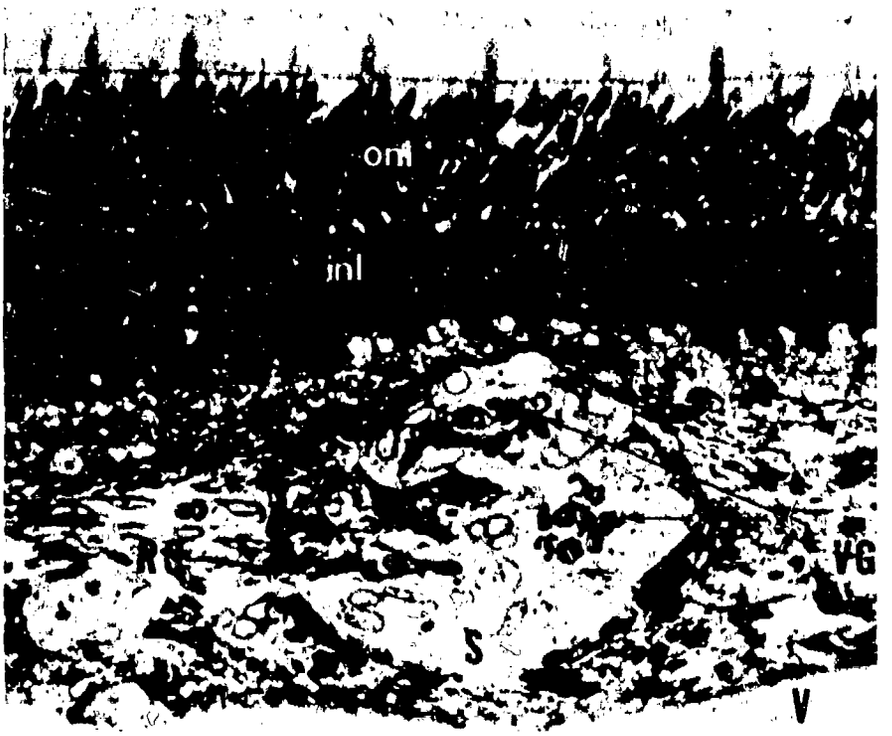

Figure 9: A photomicrograph depicting the dichotomy that existed in the nerve fiber layer of the temporal retina of infant #5 (control, 36 weeks at death, 7 weeks of oxygen administered) between the vanguard region (VG), with its stacked spindle cells, and the rearguard region (RG), with its complete vascularization. These two regions were separated by a shunt of rolled mesenchyme (S) entrapping erythrocytes in their extracellular space (⟷). Vitreous (V); outer nuclear layer (ONL), inner nuclear (INL). (X530)

rearguard side of the shunt, the extracellular spaces are transformed into a lumen surrounded by spindle cells (with decreased lipid) which are becoming morphologically endothelial in nature (characterized by: flattening, and marginal flap formation) (see Figure 10C). Outside the shunt in the adjacent rearguard region, these lumen are surrounded by mature endothelial cells, between which are interspersed a few incompletely differentiated cells that retain the morphological markers of their spindle cell ontogeny (Figure 10D).

Light Micrograph of the Optic Nerve in Case 5 (Grade III ROP)

A section of optic nerve from infant #5 (control) displays a normal population of astrocytes and neural axons. There is also evidence of a normal vascular supply from nerve head capillaries and the short posterior ciliary artery. This finding implies that the damage to the nerve fiber layer of the retina is not retrograde to the fibers of the optic nerve (see Figure 11).

Light Micrograph Depicting the Separation of Formed Vessels in the Nerve Fiber Layer of Control Versus Experimental Infants

A section of the temporal retina from control infant #5 (Figure 12A) provides a very coarse means to evaluate the width of the "capillary free zone" by measuring the separation between formed vessels in the nerve fiber layer (average separation 92μ). The experimental infant #6 was used as a comparison (Figure 12B). The separation of the formed vessels in the experimental infant's nerve fiber layer was approximately one-half (average separation 59μ) that of the control in-

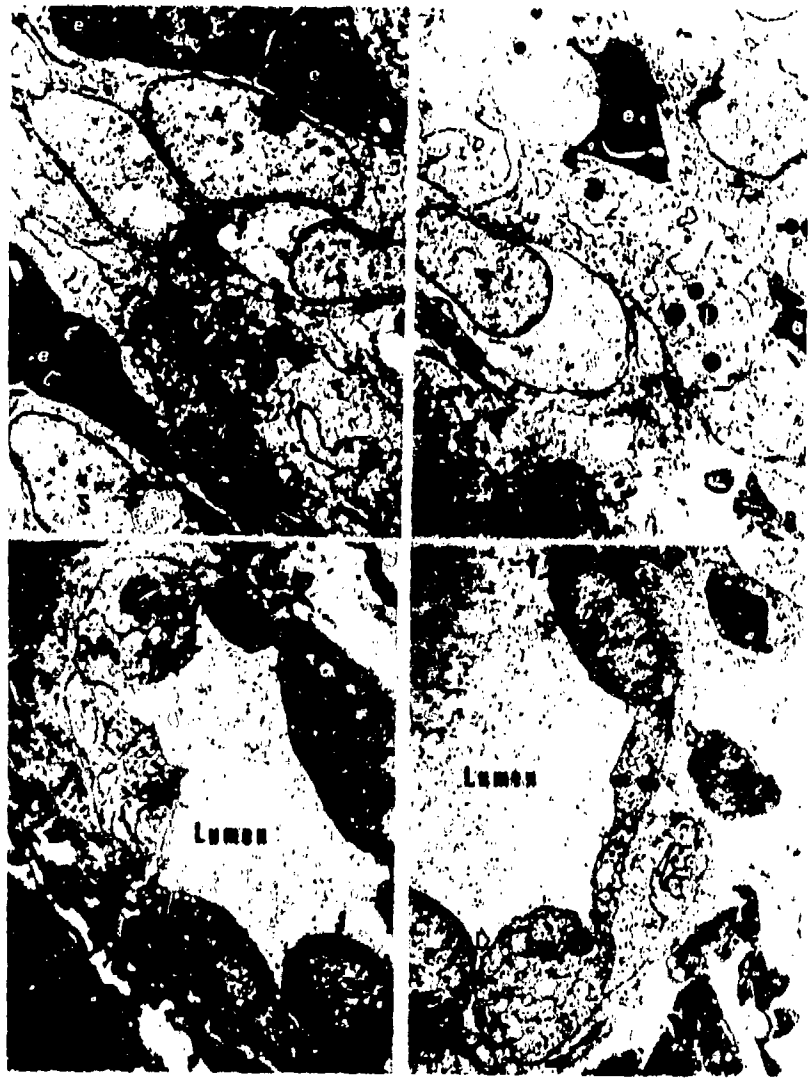

Figure 10: Electronmicrographs depicting vanguard (Figures 10A and B) and rearguard (Figures 10C and D) regions in the nerve fiber layer from control infant #5 (36 weeks at death, 7 weeks of oxygen administered). In the vanguard region there are extracellular erythrocytes (e) and lipid (l) is accumulated within the cytoplasm of the spindle cells (S). In the rearguard region the extracellular spaces are transformed into lumen surrounded by spindle cells (with decreased lipid) (l) which are transitioning into endothelial cells (te). Endothelial changes include flattening of the cell and marginal flap (f) formation. In the newly formed vascular wall lipid may be found more frequently in the endothelial cells that exhibit a rounded immature character (⟶). (X6000)

fant's measurements.

DISCUSSION

Since this histological study is derived from such a small sample (3 control and 3 experimental se.. of eyes) it is not possible to make conclusive inferences about the influence of vitamin E from these data. The histological data did conform to pre-existing theories about the pathological changes associated with the development of the disease at the light microscopic level (5, 11, 12) and will be described in this section in the same sequence that the histological examination was performed. Some of the theories concerning early

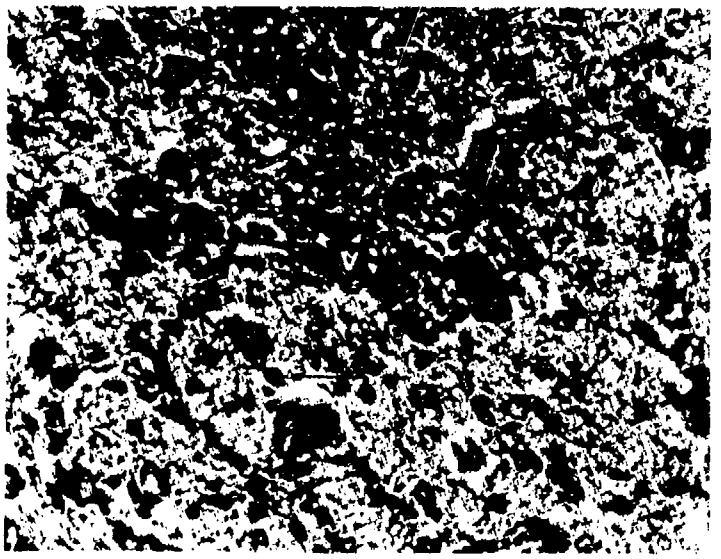

Figure 11: A light micrograph of the optic nerve from infant #5 (control, 36 weeks at death, 7 weeks oxygen administered). There is evidence of normal vascular supply (V) and a normal population of astrocytes (arrow) and neural axons (a). This finding implies that the damage to the nerve fiber layer of the retina is not retrograde to the fibers of the optic nerve. (X530)

stages of vascular development could not be confirmed because none of the infants' eyes obtained was less than 28 weeks gestation. This may explain the lack of macrophages and autophagic vacuoles (4) within the population of spindle cells (primitive mesenchymal cells).

The retina of control infant number 5 (36 weeks at death) shows evidence of proliferation and dense packing of spindle cells within the nerve fiber layer which is commensurate with stage I proliferation by the Foos classification (see Table 2). Although there was dense packing of spindle cells in the retinal nerve fiber layer there was no evidence of axonal disruption and sections of the optic nerve were normal, implying that the nerve damage was not retrograde. (Figure 11, results).

Infant number 5 also exhibited endothelial cell proliferation and shunt formation characteristic of stage II by the Foos classification. Ultimately, the infant exhibited stage III ROP (an extra-retinal proliferative stage) where the neovascularization extended into the vitreous. Intravitreal neovascularization could not be documented histologically but the infants' clinical record indicated that neovascular growth into the vitreous began the eighth week of life. Intracellular lipid accumulation is seen extensively in infant #5 (Figure 6A and B) and is confirmed with Oil-Red-O staining of frozen sections. Lipid accumulation in this disease is not commented on by Foos or Ashton--possibly because their microscopic techniques did not provide sufficient resolution to make the lipid readily discernible.

The presence of numerous pseudopodia in the spindle cells of the nerve fiber layer of control infant #5 (Figures 6A and B) may suggest the exaggerated proliferation of spindle cells, although cells in active mitotic division were not noted. The control infants exhibited small mitochondria and rough endoplasmic reticulum. Gap junctions were noted between adjacent spindle cells in the retina of control infants (Figure 7).

In contrast to the control infants, three experimental infants exhibited no junctional complexes between the spindle cells, little lipid accumulation, and normal mitochondria and rough endoplasmic reticulum. The spindle cells were very

Figure 12: A light micrograph from the temporal retina of control infant #5 (36 weeks at death, 7 weeks of oxygen administered) and experimental infant #6 (46 weeks at death, 29 weeks of oxygen administered) (Figures 12A and B, respectively). It appears from very coarse observation that the separation between the formed vessel and nearest capillary (arrows) appears to be greater in the control infant (average 92μ) than the experimental infants (average 52μ). (X530)

sparse within the vacant appearing nerve fiber layer, which is compatible with the normal developmental sequence described by Ashton (4), in which there is an initial invasion of the nerve fiber layer by spindle cells which ultimately form the endothelial-lined capillary network.

A sparse spindle cell distribution was exhibited in the oldest infant (#6, experimental, 46 weeks at death). This may indicate an antioxidant protective effect with vitamin E. High oxygen (≥80%) concentrations may prevent the continuation of retinal vascular development, and vitamin E, as a membrane constituent, may protect the developing vasculature while it is under the influence of high oxygen concentrations. The protective effect of vitamin E might presumably permit the retinal vessels to eventually resume at a more normal rate of development as the infant matures. Although

Table 2: Histological Classification of the Various Grades of R.O.P. (5)

I. Spindle cell proliferation in the nerve fiber layer (other features of normal vascular development still exist).

II. Vanguard of proliferation spindle cells with a rearguard of densely compact proliferating endothelial cells.

III. Extension of capilliaries from rearguard region through the retina and into the vitreous.

IV. Partial retinal detachment.

V. With development of the disease, at later times, the retina can be incorporated into a cicatricial mass.

infant #6 developed severe hyaline membrane disease, resulting in poor oxygen exchange in the lungs, there was sufficient oxygen stress in the first week of life to cause vasoconstriction of the retinal vessels thereby preventing new vessel growth and development.

It is reported that vascular proliferation occurs while the infant is still under oxygen therapy (13, 14). This finding contradicts earlier reports that suggest that the proliferative process was triggered after the infant was removed to a room air environment. The absence of proliferation in the developing vasculature of the experimental infants who were under oxygen therapy for prolonged periods would further strengthen the argument of the protective influence of vitamin E. It was fortunate to have a matched experimental infant (#3, 29 weeks at death, 3 weeks oxygen administered), for comparison with our experimental infant (#4, 29 weeks at death, 2 weeks oxygen administered). Each of the infants exhibited the characteristic, sparse, spindle cell pattern in the nerve fiber layer (Figures 8A and B) that would indicate that the proliferative process had not occurred. This evidence combined with the apparent protection of all the experimental infants from developing a dense population of spindle cells in the retinal nerve fiber layer would tend to substantiate the existence of a true dichotomy between the spindle cell morphology of the control and experimental infants.

Light microscopy of the retina of infant #5, (control, 36 weeks at death) depicts a clear morphological gradient between the cells that exist in the vanguard versus those of the rearguard regions (Figure 9). This gradient provides a unique opportunity to examine the spindle cell (the embryologic precursor of the vascular endothelial cell) in various stages of development. If this morphological distinction is considered in conjunction with the angiogenesis (vessel development) hypothesis proposed by Ashton (4), which suggests that the retinal vascular changes noted in ROP occur in two distinct steps, vaso-obliteration and vaso-proliferation, then cytological distinctions can be noted between spindle cells in the vanguard region and formed endothelial cells in the rearguard region. Moreover, the region of the shunt may reflect the transition of the spindle cell to endothelial cell.

It is noted that the spindle cell retains its character on the vanguard side of the shunt with little differentiation. These cells contain numerous lipid inclusions, and erythrocytes are trapped within the extracellular space (Figures 10A and B). Progression toward the rearguard side of the shunt reveals further differentiation of the spindle cells into cells exhibiting endothelial characteristics (flattening and the development of marginal flaps). These cells are observed to form a lumen through which erythrocytes pass (Figure 10 C). It was noted that lipid accumulation had diminished when transiting vanguard to rearguard. Finally, in the rearguard region the vessel is formed of endothelial cells with remnant spindle cell characteristics and lipid inclusions are only occasionally observed. (Figure 10D)

While evaluating the vascular morphological changes in this study it was noted by coarse reticle measurement that there seemed to be greater separation between formed vessels (arterioles or venules) and the nearest adjacent capillaries in the control infants than in experimental (see Figures 12A and B). This observation is merely suggestive, since it is not possible to reliably evaluate capillary free zones by this method, because of the difficulty in distinguishing artery

from vein and the possibility that a nearby capillary may not appear in any given section of tissue.

Even with these limitations our observations suggest the possibility that there may be a protective influence of vitamin E on the primitive endothelial cells next to arteries or veins, thereby permitting a more normal progression of the retraction process while under elevated oxygen in the perinatal period. This in turn might reduce subsequent vaso-proliferation as the infant matures. Future studies of the influence of vitamin E on ROP could incorporate into their protocol a suggestion to measure the size of the capillary free zone in infants that have been administered varying amounts of therapeutic oxygen. The measurements could be obtained by flat mount techniques in post-mortem tissue.

The "capillary free zone" surrounding the formed vasculature could be evaluated in vivo by fluorescein angiography (15), which may provide a means of determining the extent of vascular damage during the active stages of the disease. One important factor elucidated by Kushner (16) was the marked variability in retinal angiogenesis which may provide for variance in susceptibility noted in ROP within the premature population and with infants that are full term (17), and those infants that had not received oxygen therapy (18).

Implication and Potential Applications of Histological Observations

From these observations the angiogenesis hypothesis proposed by Ashton (12) is further substantiated. The spindle cells appear, ultrastructurally, to be the embryonic precursor of the lumenal endothelial cell. The gradient in the abundance of lipid inclusions from vanguard (high) to rearguard (low) could possibly be explained if the spindle cells have fewer antioxidant protective enzymes, possibly due to biological immaturity of the premature newborn infant. The problem is then confounded by high oxygen exposure. Accumulation of lipid droplets in the retinal pigment epithelium has been observed in albino rats kept on antioxidant deficient diets (Dratz, personal communication). Although observed in different cell types in this study the accumulation of lipid droplets noted by Dratz in experimental animals, and in the control population of premature infants in this study may indicate a link between oxidative stress and disturbances in lipid metabolism.

The destructive influence of oxygen on the maturing retinal vasculature seems to be directed at the sensitive capillary free zone. If the normally low antioxidant stores of the premature infant can be supplemented from external sources, the resultant higher concentrations of antioxidants, during the crucial vasoobliterative stage, seem to decrease the vasoproliferative response that results when the infant reaches the critical maturity (7 weeks post-partum). The process of antioxidant protection could be reflected morphologically as the normal precursor spindle cells maintaining their primitive characteristics without lipid accumulation then differentiating to endothelial cells at a normal rate (see Table 1B - results).

Hopkins (19) suggests changes in membrane permeability allows for the transfer of signal molecules (e.g., cyclic nucleotides) between cells and this may be visualized cytologically by the formation of gap junctions. It is also hypothesized (not confirmed) by Hopkins (19) that gap junctions may be important when groups of cells are required to act in concert. This hypothesis implies gap junctions may play a role in the regulation of cellular proliferation.

Kretzer (8) evaluating spindle cell proliferation in infant retinas suggests that the number of gap junctions increases, whereas Meyer (20) suggests a decrease when evaluating regenerating rat liver. Further studies of tissue from eyes of experimental infants will provide more insight into biochemical questions of lipid accumulation, whole mount preparations will provide answers concerning the influence of vitamin E on the capillary free zone while the infant was maturing under the influence of therapeutic oxygen, and electron microscopy may clarify the contribution of gap junctions to cellular proliferation.

In summary, the histological material presented above is in accord with data from other studies (5, 12), and provides insights for future ultrastructural evaluation of neovascularization (e.g., diabetes, central retina vein occlusion, or Eale's disease). The influence of vitamin E in ROP cannot be established with this histological study of six infants, but the results are consistent with the results of the clinical study (1,2). Possibly future studies would evaluate the effect on ROP of an antioxidant "cocktail" that would be designed to intervene at several points in the reaction cascade leading from the generation of free radicals to tissue damage.

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Histological evidence of retinal damage associated with the clinical observation of Retinopathy of Prematurity (ROP) grade III was documented in preterm infants receiving the minimum dosage of vitamin E recommended by the American Academy of Pediatrics (5 mg/kg/day), and exposed to high concentration/duration of oxygen at birth. Matched infants that were provided a higher oral dosage of vitamin E (100 mg/kg/day) did not develop the serious grade of retinopathy (grade III) (1,2). In this paper cytological correlates are described which substantiate pre-existing theories concerning the pathological changes associated with the development of the disease at a light microscopic level. Moreover, observations made at the electromicroscopic level permit distinctions to be

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made concerning the newly formed retinal vessels, in treated versus non-treated infants, that have not been noted in the history of this disease. These retinal distinctions suggest that vitamin E may be efficacious in reducing the severity of ROP. Lastly, a mechanism is suggested for the action of vitamin E in reducing the severity of ROP.